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AN IMPROVED SIMPLIFIED MEMBRANE FILTRATION METHOD FOR DETECTION AND ENUMERATION OF *ESCHERICHIA COLI* FOR USE IN MONITORING DISINFECTED WASTEWATER EFFLUENT

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Abstract

The USEPA recommends use of the m-TEC method for detection and enumeration of *E. coli* in chlorinated/dechlorinated effluents from Publicly Owned Treatment Works (POTW). This study compares the m-TEC with a commercially available method, COLISCAN MF™ for accuracy and ease of use for POTW laboratories. The m-TEC is based on pH indicators of organic acid production to generate a yellow to yellowish-brown color as the *E. coli* identifying characteristic. The COLISCAN MF™ differs from the m-TEC in that it contains two chromogenic substrates, which *E. coli* is capable of producing enzymes to metabolize, resulting in distinct blue pigmented colony forming units unique to *E. coli*. The study found the relationship between the two methods for enumeration to be very similar. In confirmatory tests on the COLISCAN MF™ method, 3.8% and less than 1%, false positive and false negative rates, respectively, resulted. The COLISCAN MF™ was found to be superior to the m-TEC: 1) it was substantially easier to apply because it required no resuscitation step, greatly reducing labor inputs, 2) the accuracy of *E. coli* colony counts by the technicians was much higher enhancing the NPDES compliance confidence, and 3) the overall cost for the COLISCAN MF™ was less by more than 5% compared to the m-TEC.

Key words: chromogen, COLISCAN MF™, confirmatory, *Escherichia coli*, membrane filtration

Introduction

The State of Indiana's Department of Environmental Management (IDEM) mandates through its National Pollutant Discharge Elimination System (NPDES) permitting program that

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discharges of treated wastewater effluents from publicly owned treatment works to receiving waters be analyzed for presence of *Escherichia coli* during those seasons of the year when potential for partial and full body contact with those waters exists (Indiana State Statute: IAC-2-6-1, 1989). Regulating authorities have been focusing on *E. coli* because of its specificity to fecal coliforms and its documented accuracy in indicating potential recent fecal pollution (Clark, *et al.*, 1991; Cover, *et al.*, 1992; Freier and Hartman, 1987; Kaspar, *et al.*, 1987; Mates and Shaffer, 1989).

Under its NPDES permit, the City of Elkhart's Water and Wastewater analytical laboratory conducts *E. coli* enumeration analyses using the USEPA recommended membrane filtration "m-TEC" method (Dufour, *et al.*, 1981). Laboratory technicians at the Elkhart facility have reported difficulty in differentiating among the various shades of yellow and yellowish-brown when reading the m-TEC plates for *E. coli*. Therefore, the City of Elkhart embarked on a study to compare the m-TEC method against the commercially available medium COLISCAN MF™ (Micrology Laboratories, LLC, Goshen, Indiana) for 1) sensitivity and specificity of the medium for *E. coli*, 2) the relative degree of difficulty and time requirements in preparing the media, 3) degree of difficulty in applying the respective methods, and 4) the degree of objectivity introduced in reading the plates of the two methods, including the influences of background interference due to the presence of non-coliform organisms.

As Haines, *et al.* (1993) point out, there are numerous methods for recovering *E. coli* from water. Many of these rely on organic acid production at high temperatures resulting from lactose fermentation metabolism. The m-TEC is one such method and utilizes colorimetric indicators of pH changes resulting from these acidic productions. Key pH indicators in the m-TEC agar (Difco#DF 0334-15-0) are Bromcresol purple and Bromphenol red. Recent research, however,

has shown that recovery techniques for *E. coli* are most accurate by measuring enzymatic activity (Adams, *et al.*, 1990; Brenner, *et al.*, 1996; Freier and Hartman, 1987; Kaspar, *et al.*, 1987; Olson *et al.*, 1991; Sarhan and Foster, 1990). Unlike m-TEC agar, the COLISCAN MF™ medium utilizes two chromogenic substrates, 5-bromo-4-chloro-3-indolyl-β-D-glucuronide and 6-chloro-3-indolyl-β-D-galactoside. *E. coli* has the ability to produce enzymes for both substrates while most other known coliforms produce only the enzyme for the galactoside substrate.

Procedurally, the two membrane filtration methods are quite similar, except that the m-TEC requires an initial resuscitation step for recovery of organisms that may have been injured in the chlorination process of disinfection. This initial resuscitation step involves incubation at 35°C for 2 hours to recover organisms, followed by incubation for colony growth at 44.5°C for at least 22 hours. Because the COLISCAN MF™ requires incubation only at 35°C for the entire procedure, no dedicated resuscitation step is necessary.

In reading the m-TEC plates, the technician counts as *E. coli* those colonies appearing as yellow or yellowish-brown following a urease reagent saturation of the membrane filter. Subjectivity arises in the determination of color. Consequently, it is often difficult to differentiate *E. coli* from general coliform and other background groups with confidence. For the COLISCAN MF™ plate, non-coliform colonies appear as white or translucent, non-*E.coli* coliforms appear as pink, and *E. coli* as blue. Such clear colorimetric differentiation removes a significant degree of subjectivity in judging which colonies are indeed *E. coli*.

Because of subjectivity in reading a colony with any method, there is always the issue of confirmation that the colony being read truly is the indicator organism desired. A confirmatory test attempts to independently verify the identity of those colonies from the original isolation that were questionable and therefore could result in either false positive or false negative counts. To

quantify false positive and false negative results in the colonies grown under the COLISCAN MF™ medium, an additional confirmatory study was conducted in which colonies from the COLISCAN MF™ plates were isolated and identified using an alternate method.

Methods

In the detection and enumeration study, 100 samples of chlorinated and dechlorinated effluent from the Elkhart wastewater treatment plant were analyzed over a 50-day period between July and September, 1997. Duplicates of each dilution were run (*Standard Methods*, 1995). Twenty percent of the samples were split and identical analyses were conducted at a nearby private microbiological laboratory (Micrology Laboratories, LLC, Goshen, Indiana). COLISCAN MF™ plates were incubated at 35°C, while the m-TEC plates were incubated submerged at 44.5°C waterbath for 22 hours (following the 2-hour resuscitation incubation at 35°C). All plates were read at 24 hours. Only those tests producing countable plates according to *Standard Methods* (1995) were figured into the statistical analyses.

For the confirmatory test, chlorinated and dechlorinated wastewater effluent was plated on COLISCAN MF™ medium and incubated at 35°C for 24 hours. From the resulting cultures, 236 colonies were isolated into pure cultures on agar plates. These same isolates were then cultured and incubated using the commercially available ENTEROTUBE™ identification method (BDL/Difco). For confirmation, each culture that did not match between the COLISCAN MF™ and ENTEROTUBE™ tests, was streaked on a COLISCAN gel to confirm purity.

Results

This study resulted in consistently higher *E. coli* colony counts with the COLISCAN medium than the m-TEC medium. However, the differences were found to not be significant. The counts of colony forming units were found to be very similar between the two methods as illustrates in Figure 1.

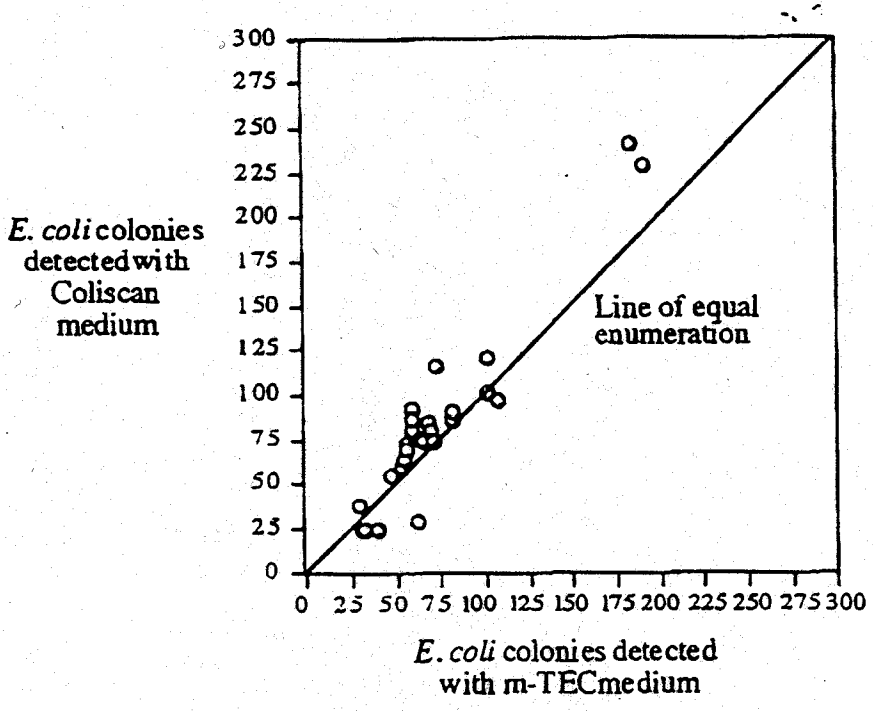

Figure 1: Comparison of COLISCAN and m-TEC media for detection and enumeration of *E.coli*

Correlation of data results between the two laboratories were relevant. Pearson coefficients for the parallel analyses on the m-TEC and COLISCAN MF™ plates run by the City of Elkhart facility and the private laboratory were 0.835 and 0.83, respectively. These results verified the quality control measures applied to study procedures. A Pearson coefficient of 0.928 was found within the Elkhart facility's correlation between the m-TEC and the COLISCAN MF™.

In this study confirmatory tests were conducted to investigate the range of false positive

and false negative readings that the COLISCAN MF™ method. For the study, the definitions of false positive and false negatives were understood as follows: 1) A false positive occurs when a colony forming unit appeared blue on the COLISCAN MF™ but tested other than *E. coli* by the confirmatory ENTEROTUBE™; 2) A false negative occurs when a colony forming unit appeared pink on the COLISCAN MF™ but tested as *E. coli* by confirmatory ENTEROTUBE™.

For the confirmatory test, of the 236 colonies picked and isolated to pure cultures from the COLISCAN MF™ membrane filter, 105 colonies were blue and therefore identified as *E. coli*. Of these 105, 94 were confirmed as *E. coli* using the Enterotube, 7 resulted in no ID, and 4 resulted in a false positive reading. The ENTEROTUBE™ confirmatory method identified these four as *Klebsiella*. (Certain strains of *Klebsiella*, *Shigella*, and *Salmonella* are known to produce the enyzme glucuronidase (Sarhan and Foster, 1991), which the COLISCAN MF™ holds specific to *E. coli*.). Therefore, the false positive rate for the COLISCAN MF™ medium was 3.8%. This compares to false postive rates of 13% found for sewage tretament plant effluent using the m-TEC by Dufour, *et al.* (1981).

The remaining 131 colonies from the COLISCAN MF™ membrane filter were pink. The expectation from the COLISCAN MF™ would be that these are general coliforms. Indeed, of these 131 pink colonies, 126 were confirmed as general coliforms by the ENTEROTUBE™ confirmatory , 4 resulted in no ID, and 1 was a false negative, resulting in a false negative rate of 0.8%. The majority of the general coliforms were identified as members of genera *Klebsiella*, *Enterobacter* or *Citrobacter*. (It should be noted that "no ID" merely indicates that the organism did not fall within the code designated by the Enterotube for either *E. coli* or a general coliform. It does not necessarily mean that the original designation is incorrect).

It is important to note that for total accuracy in confirmatory testing, colonies picked

directly from a membrane filter cannot be assumed to be pure unless streaked on to the surface of a differential medium such as COLISCAN gel or EMB.

Our initial direct picks of blue colony forming units from the membrane filter indicated that approximately 30% of the apparently well separated blue (from pink coliform colonies) *E. coli* were "contaminated" by cells from adjacent pink coliform colonies. This phenomenon has no effect on the accuracy of the COLISCAN MF™ method as the blue color of the *E. coli* overrides any additional pink from a contaminating coliform. Also, the problem is not as apparent in the pink coliforms as the presence of a (blue) *E. coli* would be obvious. This can be easily explained by possible motility on the wet membrane, or by cells originally so close that they grow as one colony forming unit. (This is less likely, however, when the number of colony forming units is small over the membrane). Therefore, this finding is important from a confirmatory point of view. However, from an enumeration perspective, this fact is irrelevant because the end result is that if *E. coli* is present, it is still correctly counted.

Discussion

The Elkhart Public Works Laboratory staff found the COLISCAN MF™ membrane filtration method to be superior to the currently practiced m-TEC method for several reasons. First, the COLISCAN MF™ consistently gave higher *E. coli* counts than the m-TEC method with greater accuracy. Reading of plates was significantly simpler for the technicians with much higher levels of confidence for *E. coli* detection than with the m-TEC plates. The technicians found it much easier to differentiate among the blue, pink, and white colonies on the COLISCAN MF™, than to differentiate between the yellow and yellowish-brown colonies on the m-TEC plates. This was

despite the fact that "bleeding" of colony growth was seen on approximately 30% of the plates in both methods. For the m-TEC trials, this bleeding was indicative of high acid production, which in turn caused yellowish coloration of numerous background organisms (the majority of which were found to be *Pseudomonas* spp.). This resulted in higher *E. coli* counts, increasing the false positive rates.

This was not the case, however, with the colonies exhibiting bleeding on the COLISCAN MF™ plates. Although the lower incubation temperatures allowed for growth of additional background flora, the *E. coli*. colonies remained distinct in their blue color and were not inhibited or suppressed by the additional background flora. (It should be noted that because wastewater effluents typically contain high amounts of background flora, background can be controlled through the use of antibiotic additives, such as Cefsulodin. Cefsulodin is effective in the eliminating galactosidase positive, non-coliform background organisms, such as *Aeromonas* spp. However, in the case of COLISCAN MF™, because of the presence of the two chromogens resulting in distinct pigments of blue, pink and white--with the white being the background--addition of an inhibitor like Cefsulodin is unnecessary for this application).

Secondly, the Elkhart Public Works Laboratory found a potential cost savings in the materials required for the COLISCAN MF™ process and the cost of the labor involved in preparing the plates over that required for the m-TEC. Projected on an annual basis, it was found that this savings was greater than 5%.

Probably most significantly, however, was the level of confidence in the detections and enumerations of the *E. coli* that the Elkhart Public Works Laboratory staff found. The City of Elkhart's current NPDES permit limits the number of *E. coli* colonies in its final treated wastewater effluent to 235 colonies/100 mL as a maximum daily limit and 125 colonies/100 mL as a

monthly average as computed via a geometric mean. It is likely that as this permit is updated and renewed, these limits will become more stringent. Using the m-TEC, it is normal practice for technicians to overcount *E. coli* on the plate because of the difficulty in differentiating yellow and yellowish-brown colonies. Though a conservative practice, it may jeopardize current and future permit limits because of high false positive rates. Additionally, high false positives do not give clear indications of the disinfection process performance.

The COLISCAN MF™ method, on the other hand, essentially eliminates overcounting because of the more precise differentiation of the *E. coli* colonies. Because of the confidence that can be placed in the enumerations, one has a much clearer definition of the performance of the treatment plant's disinfection process. This clearer definition of performance allows one to make process adjustments more proactively.

Conclusions

The authors found the COLISCAN MF™ membrane filtration method to be superior to the recommended standard USEPA m-TEC membrane filtration method for detecting and enumerating *E. coli*. The COLISCAN MF™ produced consistently lower false negatives and positives, indicating greater sensitivity with the dual enzyme substrate chromogens than with pH and urease indicators required by the m-TEC. Additionally, the COLISCAN MF™ medium took less preparation time, and was cheaper in overall material costs. Finally, the confidence developed by the laboratory technicians for detecting and enumerating *E. coli* colonies while using the COLISCAN MF™ method carries permit ramifications for the City of Elkhart's publicly owned treatment works facility.

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